DESIGN OF A SPECIFIC, SENSITIVE ASSAY FOR THE DETECTION OF ANTI-VIMENTIN ANTIBODIES USING LUMINEX BEAD-BASED TECHNOLOGY: CHARACTERIZATION AND DISCUSSION OF ANTI-VIMENTIN ANTIBODY IN THE CARDIAC ALLOGRAFT TRANSPLANT POPULATION

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Abstract

Background: The intermediate filament vimentin is not normally exposed to the immune system, however when exposed, anti-vimentin antibodies (AVA) can be provoked. AVA have been implicated in the development of cardiac allograft vasculopathy (CAV), limiting the long-term benefits of cardiac transplantation. Our aim was to develop a more specific and sensitive assay for AVA than currently available and determine if low levels of AVA are associated with CAV.

Methods: A multiplexed assay for AVA was designed and validated by parallel testing with a commercially available ELISA for AVA. Sera titration and competitive inhibition with soluble vimentin were used to assess sensitivity and specificity. Pre- and post-transplant sera from forty-six patients were tested under IRB approval. Post-transplant sera were obtained within 12 months after transplantation.

Results: In parallel titration studies, the bead-based assay was found to be twice as sensitive as the commercially available ELISA. Competitive inhibition studies of five sera resulted in a mean of 60%±28% reduction of antibody binding, confirming the Luminex assay specificity. The incidence of AVA in pre-

transplant sera was 39.1%.

Conclusions: A Luminex® bead based assay for AVA was developed that is both specific and twice as sensitive as a commercially available ELISA. Although a high incidence of AVA among cardiac transplant candidates was observed pre-transplant, longer term studies will be needed to confirm any association with CAV post-transplant.

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Introduction

The intermediate filament vimentin is a large component of mesenchymal cells that compose the aortic and pulmonary arteries of the heart. Vimentin is not endogenously exposed to the immune system, however when exposed, the immune response can elicit Anti-Vimentin Antibody (AVA). Antibodies have long been a contraindication to transplantation and up until recently only alloantibodies, predominantly HLA specific, were thought to be relevant.[1] Recent studies have focused on the development of non-HLA antibodies during the post-transplant period and their correlation to organ allograft rejection.[2,3] The development of highly specific antibody detection methods has progressed rapidly in the last ten years [1], however non-HLA targets have lagged behind.

Rapid and specific solid organ post-transplant antibody monitoring can be accomplished using Luminex® based technology or flow cytometry. The Luminex® 100/200 platform is a fluorometer; a system based on flow cytometry principles.[4] This method was proposed due to the accurate, reproducible, and rapid use in detection of anti-HLA antibodies.

Post-transplant cardiac patients can generate autoantibodies to vimentin that cause accelerated rejection in the cardiac transplant population. [5] The progression of Transplant Associated Coronary Artery Disease (TxCAD) and

Cardiac Allograft Vasculopathy (CAV) has been associated with the presence of AVA, limiting the long-term benefits of cardiac transplantation.[3] TxCAD and CAV are characterized by a complex interaction of immunologic and non-immunologic factors, which result in coronary obstruction. CAV is currently the most frequent cause of morbidity and mortality post orthotopic cardiac transplantation.[6] The development of AVA could indicate a higher risk for CAV development and graft dysfunction. By monitoring the presence of AVA in post-transplant recipients, the mortality of CAV could decrease.

In this analysis, I describe the development of a highly sensitive assay to detect AVA and discuss the incidence of AVA within the transplant heart population.

Literature Review

Vimentin

The cellular cytoskeleton is composed of five different classes of intermediate filament (IF) proteins. Vimentin (57 kDa), is a class III IF, and is found in cells of mesenchymal origin (endothelial cells, smooth muscle cells and leukocytes) and aids in structural integrity. Two vimentin monomers compose the smallest functional vimentin unit as a coiled-coil dimer. This unit is the building block for vimentin polymer assembly. [1,2] In addition to structural integrity, vimentin is important in cytosolic organelle position and is not intrinsically expressed on the extracellular surface. Vimentin is also expressed near proliferating or migrating smooth muscle cells, in the tunica intima of blood vessels. This layer of cells is composed of endothelial cells (vascular endothelial cells) and is in direct contact with the blood supply.[3]

Organ damage and vimentin exposure

Organ tissue experiences trauma throughout the transplant process, which leads to apoptosis and necrosis post transplantation. [3] The mechanism of vimentin exposure to elicit an antibody response is not fully understood; however, recent studies indicate activated platelets, monocytes and lymphocytes

can secrete or express vimentin on the extracellular surface.[4-7] The presence of vimentin within the extracellular space provides an initiation step in the development of AVA. The action of AVA binding to surface vimentin on circulating leukocytes induces the release of tissue factor, formally known as thrombokinase, expression of P-selectin, vimentin and additionally, tissue factor by platelets, resulting in the formation of platelet:leukocyte (P:L) conjugates. P:L conjugates have an increased ability to adhere to activated endothelial cells and elicit T cell activation through release of tissue factor, enhancing the exposure of vimentin to the immune system.[7] Damaged cells that undergo apoptosis have also been observed to express vimentin on the extracellular surface. [8-11] Current evidence would support two mechanisms of vimentin exposure leading to transplant associated coronary artery disease:

A) Apoptotic endothelial cells (donor origin)

B) Circulating activated lymphocyte cascade (recipient origin)

Coronary Allograft Vasculopathy and Endothelialitis

Coronary allograft vasculopathy (CAV) is the leading cause of morbidity and mortality post orthotopic heart transplantation, found in 42% of patients five years post-transplant.[12] The invasive coronary angiography is considered the

standard for CAV diagnosis, however intravascular ultrasound can also be used to determine the thickness of a transplanted artery.[13] Unfortunately, re-transplantation is the only definitive cure for CAV. The true underlying cause of CAV is still unknown; however it is thought to be an immunologic phenomenon, resulting from an inflammatory response, leading to smooth muscle cell proliferation and coronary obstruction. A recent study examined cellular infiltrates from the vessel walls of a patient with CAV and indicated a distinguished presence of T-cells localized in the neointima and the adventitia.[14] This particular cell population is associated with endothelialitis, and localization associated with chronic rejection. Endothelialitis is a constant state of endothelial cell inflammation in the allograft vascular wall which can lead to accelerated atherosclerosis.[15] The culmination of these immunologic events can result in vascular permeability (of immunological infiltrates) and endothelial injury (perhaps exposing vimentin to extracellular spaces). It is believed the continued presence of tissue-specific immunity (anti-intermediate filament antibodies) could contribute to

CAV development.[16-19]

Endothelialitis can activate smooth muscle cells to proliferate and migrate to the intima as an effect of injury.[20] The smooth muscle cells produce cytokines during proliferation (e.g., TNF-α) which have been shown to trigger secretion of vimentin from monocytes. Elevated TNF-α levels have been associated with CAV.[5,21] Additionally, ischemic reperfusion injury can cause stress to the tissues, resulting in pro-inflammatory cytokines correlated with CAV development. The injury to the endothelial cell layer and activated smooth muscle cells may result in the exposure of vimentin to the extracellular surface and generation of anti-vimentin antibody[21].

Rejection in the cardiac allograft

Donor specific antibodies (DSA) have been accepted as a contraindication to solid organ graft survival [22,23]. Antibody mediated rejection (AMR) occurs via the humoral immune response upon DSA binding to the donor heart endothelium. The most egregious antibodies are directed to mismatched HLA antigens and can pre-exist or develop de novo post-transplant. The immune response causing AMR is activated predominantly through the classical pathway of the complement cascade.[24] Through this mechanism, downstream complement complexes form a membrane attack complex (MAC) and initiate cell lysis.

Complement activation can occur without cells lysis, concluding in endothelial cell activation and chronic inflammation.[25] Complement independent mechanisms can also activate endothelial cells via direct cross-linking of HLA on the endothelial cell surface. As discussed in the progression of CAV, activated endothelial cells will produce growth factors (fibroblast growth factor, platelet derived growth factor, cytokines and adhesions molecules) aiding the inflammation. The inflammation can be further aided via interaction of immune effector cells (macrophages, neutrophils and natural killer cells) and Fc receptors.[26] The cumulative effect of complement dependent and independent immune functions can result in injured or activated endothelial cells and CAV development.[27-29]

The role of non-HLA antibodies, such as AVA, in the development of AMR is an active area of research, and non-HLA antibodies are also likely capable of injuring the allograft via complement dependent and independent mechanisms. AMR can result in graft dysfunction, worsen graft survival and result in a higher incidence of CAV.[27,30] Until a recent meeting held in 2010 by the International Society for Heart and Lung Transplantation (ISHLT), the diagnosis of AMR was unstructured across transplant centers. This presented difficulty when assessing the impact of AMR on the patient population. Since 2010, AMR

has been defined by "histopathological and immunophenotypic criteria and does not require clinical dysfunction or serological evidence of donor-specific antibody production."[31] Defining AMR is important because historically a major criterion of AMR was the presence of HLA-specific DSA even though AMR may occur in the absence of HLA-specific DSA.[32]

Anti-vimentin antibody detection methods

Solid phase immunoassays including indirect immunofluorescence, ELISA and western blot have been used for detection of anti-vimentin antibody over the past 25 years[17,19,30,33]. Anti-vimentin antibody has long been a focus of auto-immune diseases[19]; however, it has recently been correlated with CAV.[9,12,17,28,31,34,35] Antibody detection methods have changed since researchers began identifying alloantibodies. Previous to laser technology, complement dependent lymphocytotoxicity (CDC) testing of sera was used to evaluate the presence of donor reactive antibodies. The CDC test lacked consistency and was subjective. To enhance specificity for certain antigens, the enzyme linked immunosorbent assay (ELISA) was adopted and later used to access anti-vimentin antibodies[17,19]. This assay used a plate coated with vimentin protein. The serum incubated on the plate and antibody binding was detected generally with a horseradish peroxidase-conjugated anti-human IgG antibody. Addition of

the enzyme substrate, O-Phenylenediamine dihydrochloride, resulted in an enzymatic reaction indicative of primary antibody binding. The reaction results in a color change which is measured at a specific optical density. [18] Unfortunately, the ELISA method is unable to detect very low-level antibodies present in the serum. However, the Luminex® fluoroanalyzer can detect antibodies well below that of ELISA. While flow cytometry can also be used to detect anti-vimentin antibody [36], it is more time consuming. With an ability to detect lower level concentrations, the presence of antibody could be detected before it becomes clinically symptomatic. The Luminex® technology is currently being used in many transplant immunology laboratories, mainly due to its accuracy, reproducibility, and high throughput.

Luminex Technology

Early detection of low-level antibodies in post-transplant recipients is vital to graft function due to the effects that rejection can have on the organ tissue. The Luminex® Fluoroanalyzer screening technology has brought a highly sensitive and specific instrument to the frontline of solid-phase immunoassay development. Rapid and specific solid organ post-transplant antibody monitoring can be accomplished using Luminex® based technology.

The Luminex® 100/200 platform is a fluorometer; a system based on flow cytometry principles. The analyzer uses two lasers, the first to detect microspheres that are impregnated with a unique combination of two dyes and coupled with different proteins. The second laser identifies the secondary detection antibody. The sample flows through a sheath delivery system where the antibodies bound to the beads are detected by the lasers. This method was proposed due to the accurate, reproducible, and rapid use in detection of anti-HLA antibodies.[37]

Post-transplant cardiac patients can develop autoantibodies to vimentin. The presence of AVA has been reported to cause accelerated rejection in the cardiac transplant population. [17,30,34-36] The progression of CAV has been associated with the presence of AVA, limiting the long-term benefits of cardiac transplantation. By monitoring the presence of AVA in post-transplant recipients, clinical intervention could decrease the mortality of CAV. Post-transplant monitoring for AVA could allow detection of low-level antibodies, before they can lead to graft dysfunction. Using the Luminex platform and seroMAP bead technology, we produced a sensitive detection method, which has higher detection of AVA over that possible with the current commercially

available ELISA kits. This assay can facilitate post-transplant monitoring of cardiac transplant recipients to evaluate the presence of AVA and provide clinicians with the knowledge of the increased risk for the development of CAV.

Materials and Methods

Patient study group and samples

Sixty-three patients who received orthotopic cardiac allografts at Johns Hopkins Hospital were analyzed in this IRB approved study (NA_00077525). The serum samples were chosen on accessibility, receipt of heart transplant and availability of clinical data. All serum samples were stored according to laboratory protocol to preserve reactivity. All serum specimens were obtained for routine clinical testing.

Acute rejection

Routine endomyocardial biopsies were performed post-transplant. Biopsies were evaluated for acute rejection and graded with the 2005 International Society of Heart and Lung Transplantation (ISHLT) criteria.[6]

Multiplex assay bead activation and protein coupling

Recombinant human vimentin (R&D Systems, Inc., Minneapolis, MN, USA) was attached to Luminex® SeroMAP™ microspheres using the manufacturer provided procedure, "Sample protocol for two-step carbodiimide coupling of protein to carboxylate microspheres".[4] Addition of the vimentin protein was titrated using 1ug, 5ug, 25ug and 125ug amounts of protein and

evaluated using phycoerythrin (PE)-conjugated rat monoclonal anti-human vimentin antibody (R&D Systems, Inc., Minneapolis, MN, USA). This process was repeated using Human IgM Purified Immunoglobulin (Sigma-Aldrich, Inc., MO, USA) and detected with phycoerythrin (PE)-conjugated goat polyclonal anti-human IgM antibody (Life Technologies, Frederick, MD, USA). A naked bead was activated using the same protocol, with no protein attachment to serve as an internal negative control for non-specific binding (Table 1).

Bead	Purpose
1-Human IgM	Positive Control
2-Recombinant Vimentin	Detection of anti-vimentin antibody
3-no antigen	Assess the background of the assay

Table 1. Luminex multiplex test composition.

Control sera

Control sera were comprised of a commercially available NAB (pooled normal AB blood-type non-sensitized male sera) and a Pooled Positive Control (PPC). The PPC was made by pooling sera from patients confirmed positive on the ELISA assay.

Luminex multiplex assay procedure

Sera were incubated with the micro-bead suspension and PBS-TBN (.1%BSA, .02% Tween-20, .05% Azide, pH7.4) at a 1:25 dilution on a Multiscreen® filter plate in the dark for 30 minutes at 22 degrees Celsius. Following incubation, five washes of 250uL using PBS-TBN were performed. A secondary antibody, Phycoerythrin (PE)-conjugated goat polyclonal anti-human IgM antibody, was added at a 1:25 dilution and incubated in the dark for an additional 30 minutes at 22 degrees Celsius. Binding of AVA antibodies were detected using the Luminex® 100/200™ fluorometer platform. Results were reported as median fluorescence intensities (MFI).

Semi-quantitative ELISA assay procedure

The ELISA was performed according to the VIDIA's instructions. Absorbance was measured at 450nm using an Opsys MR™ Microplate reader and reported as optical density.

Test results interpretation

To control for inter-assay variation, test results were normalized to the negative control (NAB) as ratios of the test bead MFI to NAB test bead MFI. A Luminex ratio greater than 1.25 was interpreted as positive. The ELISA sample positivity was determined by the manufacturer to be a ratio greater than 1.34.

Competitive inhibition by blocking

Serum was titrated at 1:5, 1:10, 1:25 and 1:50 then incubated with 2ug soluble vimentin for 10 minutes at 4 ^{o}C and 20 minutes at 22 ^{o}C prior to being run on the Luminex assay.

Statistical Analyses

Differences in distribution were assessed by the chi squared and Fischer's exact test. A p value ≤ 0.05 was considered statistically significant. Test variability was determined using the coefficient of variation. A coefficient of variation ≤ 0.10 was considered acceptable.

Results

Titration of protein coupling

A titration suggested by Luminex® using varying amounts of Human Recombinant Vimentin and IgM was performed to determine the optimal amount of protein for each bead. The vimentin test bead was optimized at 125ug and detected with phycoerythrin (PE)-conjugated rat monoclonal anti-human vimentin antibody (R&D Systems) and the IgM positive control was optimized at 5ug and binding verified with phycoerythrin (PE)-conjugated goat polyclonal anti-human IgM antibody (Life Technologies). (Figure 1, page 17)

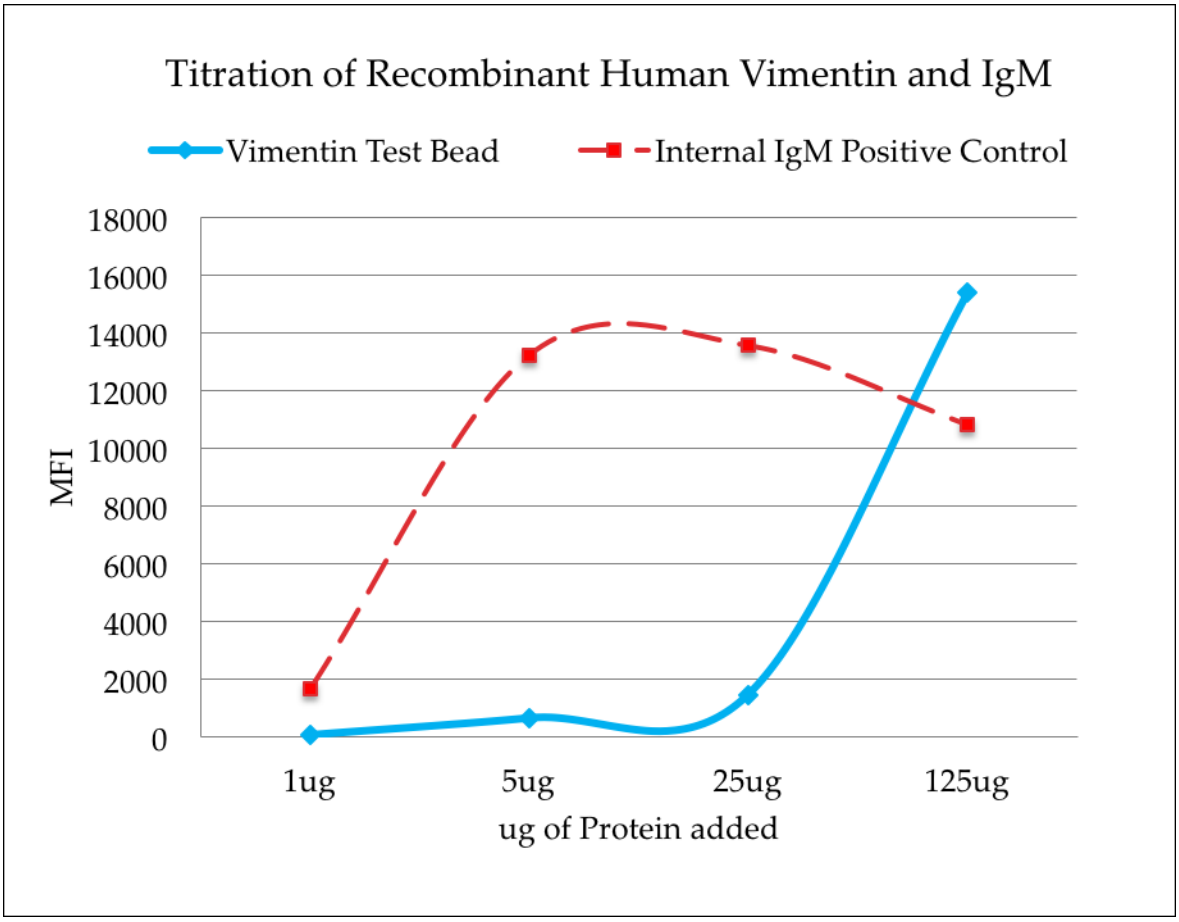

Figure 1. Protein coupling titration. The blue line measures the vimentin test bead MFI and the red dashed line measures the internal IgM positive control MFI. The vimentin titration could not be completed but due to the comparable size of the vimentin protein and HLA, however, the saturation levels observed using 125ug of vimentin are approaching saturation levels we observe on HLA Luminex assays and therefore the use of 125ug was considered comparable.

Titration of secondary detection antibody

The secondary antibody, Phycoerythrin (PE)-conjugated goat polyclonal anti-human IgM antibody (Life Technologies) was titrated from 1:25 to 1:1000 to optimize results. The experiment was carried out following the first incubation, of beads and sera, for 30 minutes at 22 degrees Celsius. The signal was maximized at 1:25, a level comparable to commercial Luminex bead based tests.

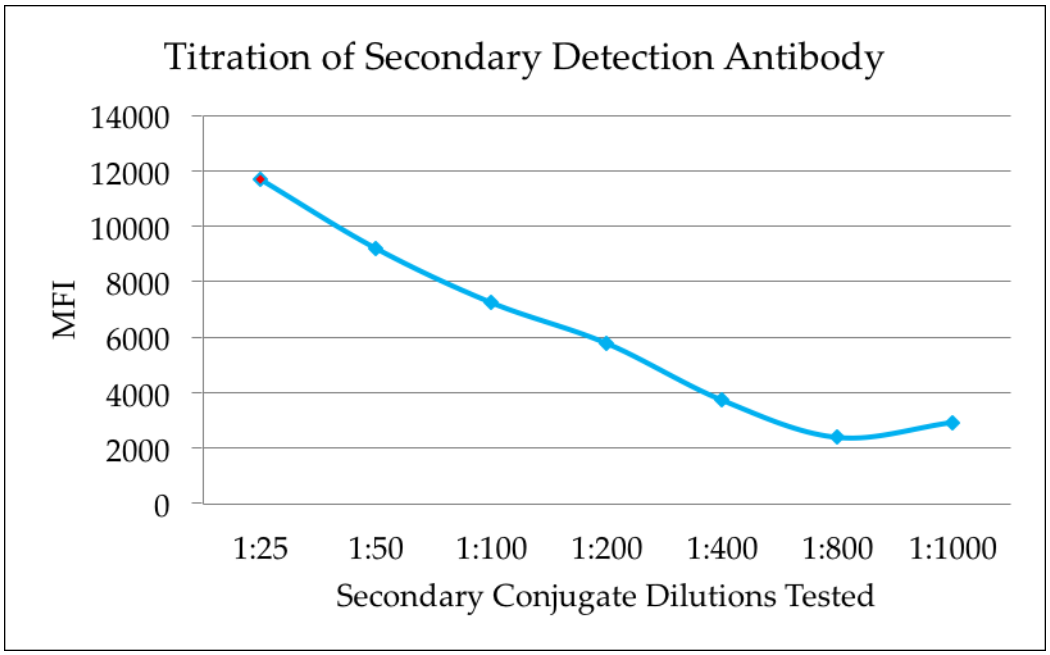

Figure 2. Secondary antibody titration was performed using NAB serum and PE-conjugated anti-human IgM antibody. The reactivity fell off quickly and linearly. The detection antibody was used at 1:25, indicated with the red marker.

Analyses of test reproducibility

In an effort to evaluate test reproducibility the NAB and the Pooled Positive Control (PPC) sera were tested in quadruplicate on the Luminex assay. The coefficients of variation were 0.07 and 0.10, respectively. For an assay with the sensitivity of Luminex, a coefficient of variation less than or equal to 0.10 was considered acceptable. It was important to establish the reproducibility of this test for interpretation and quality control purposes. (Figure 3)

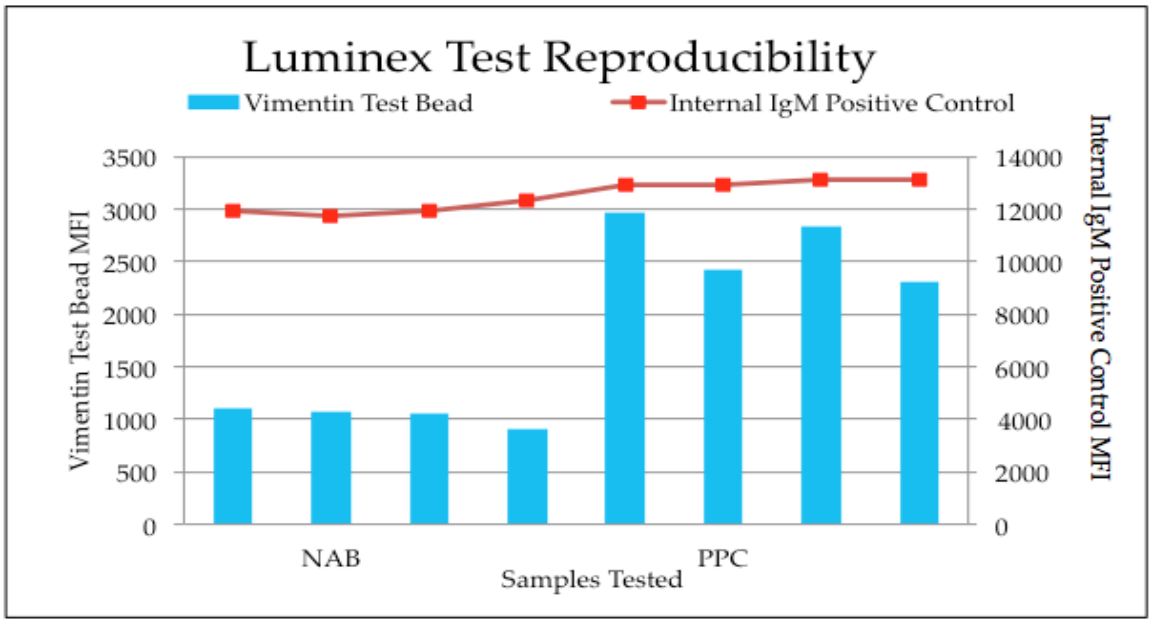

Figure 3. Analysis of test reproducibility. The blue bars measure the vimentin test bead MFI. The internal IgM positive control bead was graphed across samples to ensure comparable detection levels (StDev= 554MFI).

Optimization of test sensitivity

To ensure we were observing the optimal signal-to-noise ratio the PPC was titrated from 1:5 through 1:2000, as indicated in Figure 4. The vimentin test bead signal to the naked bead signal determined the signal-to-noise ratio. The ELISA kit controls were tested as a reference. The signal-to-noise was optimized at 1:25, with a decrease in signal at 1:50. All sera were tested at 1:25.

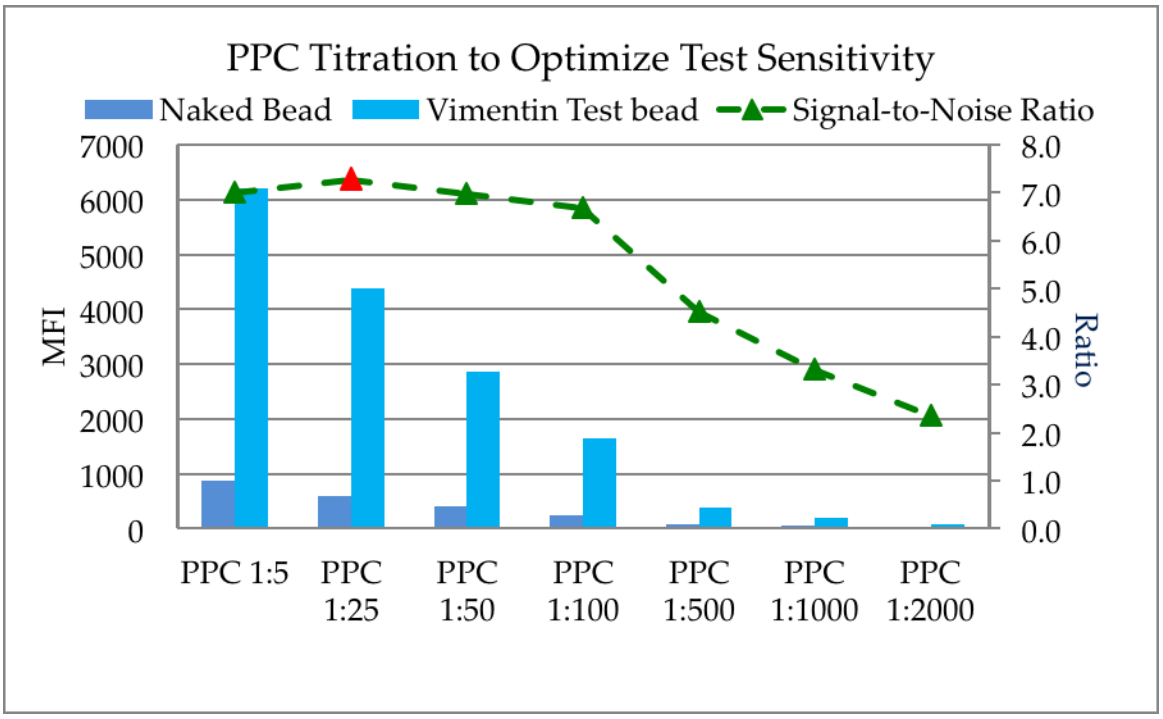

Figure 4. The PPC and the ELISA kit controls were tested. The light blue bars measure the vimentin test bead MFI. The blue bars indicate the naked bead MFI. All MFIs are measure on the left y-axis. The signal to noise ratio is indicated by the dashed line and are measured by the right y-axis.

Assay comparison

To compare sensitivity levels, results from a commercially available ELISA kit were correlated with those from the Luminex multiplex assay. Fifty-seven sera from heart patients were tested in parallel. The Luminex sample positivity was normalized to the NAB as ratios of the serum test bead MFI to NAB test bead MFI. The distribution of negative and positive samples was not statistically different however as evident in the upper left quadrant, there were numerous samples positive by Luminex that were negative by ELISA.

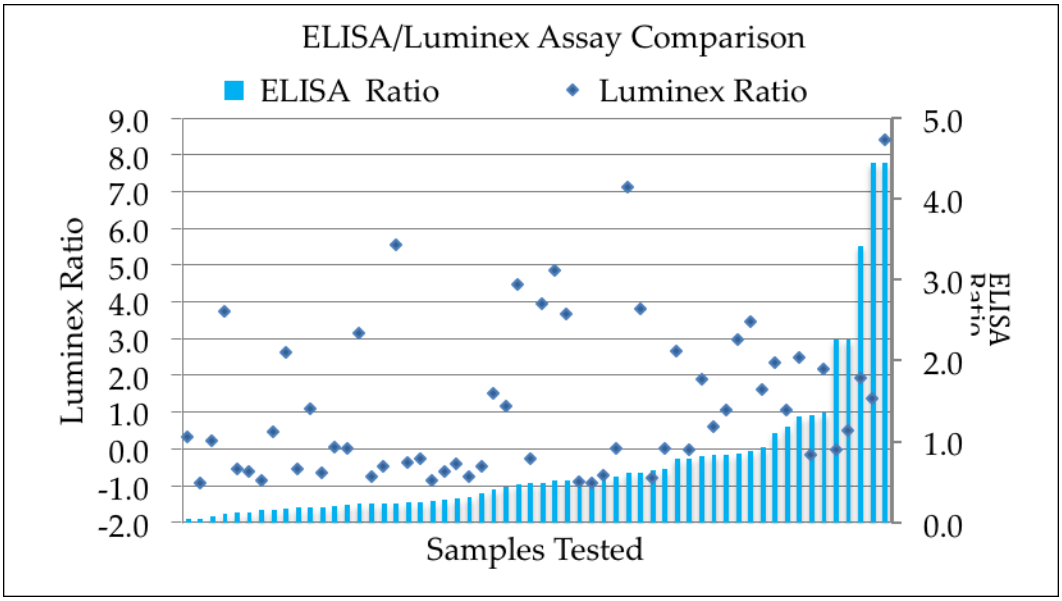

Figure 5. Multiplex assay comparison. The vertical dashed line indicates the positive ELISA threshold and the horizontal dashed line indicates the positive Luminex threshold. Chi-squared is equal to 2.379, p-value=0.12, R=.202.

Competitive inhibition using soluble vimentin

To confirm the Luminex positive only sera were detecting anti-vimentin antibody, a competitive inhibition test using 2µl soluble vimentin was performed. The test indicated an observed 95% inhibition at the lowest serum dilution, as indicated in figure 6.

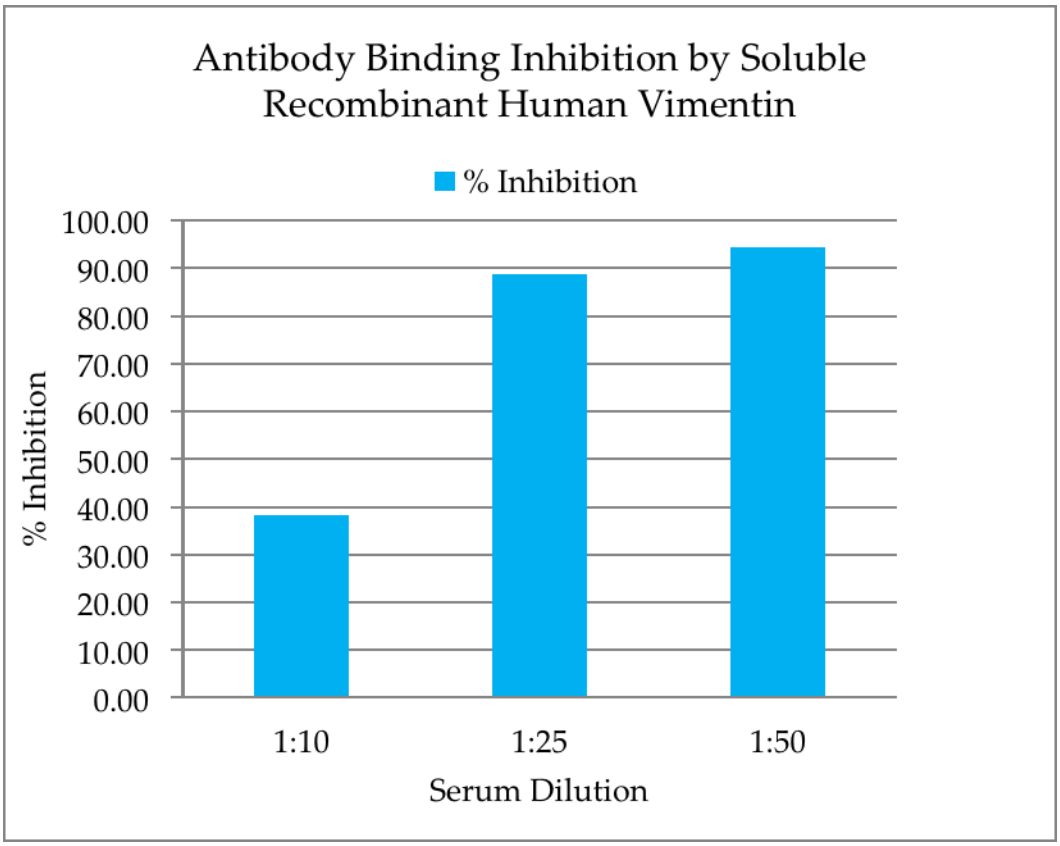

Figure 6. Competitive inhibition experiment. The y-axis measures the percent blocking observed with each dilution tested, indicated by the blue bars.

Competitive inhibition studies: continued

Competitive inhibition studies were performed at a 1:50 dilution to confirm the antibodies detected by the Luminex® AVA assay in sera that tested negative by the standard ELISA assay were specific to vimentin and the results obtained previously were not unique to the specific serum sample. The 1:50 dilution was used due to the observed inhibition in previous experiments. Five sera were tested and demonstrated inhibition of the signal with a mean inhibition of 60% and a standard deviation of 23.85.

Sample	MFI blocked	MFI unblocked	% Inhibition
1	189	1108	82.9
2	362	555	34.8
3	718	993	27.7
4	301	1154	73.4
5	149	806	81.5
Mean ± SD	343.5 ± 202	923.1 ± 220	60 ± 23.8

Table 2. Competitive inhibition study of ELISA negative, Luminex positive sera. Five sera were tested. The unblocked and blocked raw MFI are shown. The % inhibition is a ratio of the unblocked MFI minus blocked MFI divided by the blocked MFI.

Serial dilution sensitivity trial

The sensitivity of the Luminex® AVA assay was evaluated by titration of the endpoints of detection of AVA in parallel tests of serial dilutions of sera documented to be positive in the standard ELISA assay. Detection of AVA in the Luminex® assay in dilutions that tested negative in the ELISA assay helped establish the degree of increased sensitivity. (Under normal test conditions the ELISA is tested at 1:100 and the Luminex® assay at 1:25). Sample 1 and 2 reactivity remained detectable by ELISA through the 1:400 dilution, while both samples AVA reactivity remained detectable by the Luminex® assay through the 1:800 dilution. With these data the Luminex assay is observed to have a two-fold increase in sensitivity over the ELISA. (Figure 7, page 25)

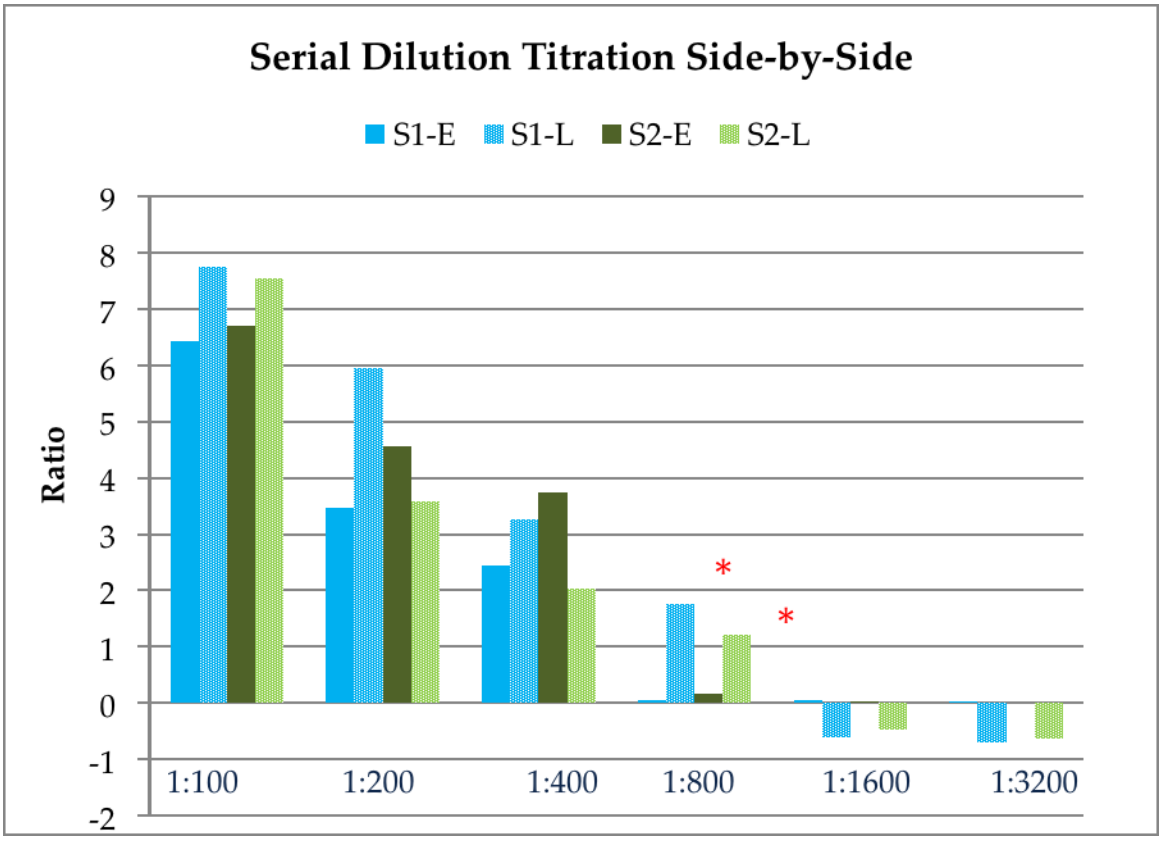

Figure 7. Parallel test results of two serum samples were run. S1 and S2 refer to samples 1 and 2. ELISA results are designated with "-E" and Luminex results with "-L". The ELISA and Luminex Ratio are plotted on the left y-axis. The luminex assay was shown to be twice a sensitive as that of the ELISA, as indicated by the asterisk.

Cardiac transplant recipient characteristics

The study group consisted of 63 patients: 35 (55%) females and 28 (44%) males. Patient characteristics are listed in Table 3. The overall incidence of CAV was 8/63. There was no correlation with race or gender.

Gender			*Coronary Allograft Vasculopathy (CAV)*	
Male		28	CAV Positive	8
	African American	9	AVA +	2
	Caucasian	18	AVA-	6
	Asian	1		
	Hispanic	0	CAV Negative	42
			AVA+	13
			AVA-	29
Female		35		
	African American	19	No CAV data Available	13
	Caucasian	12	AVA+	9
	Asian	3	AVA-	4
	Hispanic	1		

Table 3. Patient characteristics are listed in the left column. CAV status and AVA result are shown in the right column. There was no AVA correlation with gender, race or CAV status.

Due to possible vimentin exposure from tissue damage and AMR, HLA antibody was evaluated. 30/63 patients were sensitized to HLA class I antigens and 25/63 patients were sensitized to HLA class II antigens. 26/63 were un-sensitized to HLA. Additionally the presence of HLA donor specific antibody was trending towards significance when correlated with AVA positivity.

	HLA- Specific Antibody			
	Class I (p=. 34)		Class II (p= .28)	
	CI -	CI+	CI-	CI+
AVA-	20	15	23	12
AVA+	13	15	15	13

	Donor Specific Antibody (p=0.05)	
	DSA-	DSA+
AVA-	28	7
AVA+	16	12

Table 4. HLA sensitization (n=63) was evaluated. The presence of HLA specific antibody did not correlate with the presence of AVA, however, DSA positivity and the presence of AVA was treading toward significance.

The presence of AVA was further evaluated by time point post-transplant. Forty-six of the 63 patients had a pre-transplant sample and at least one sample post-transplant within 1 year. A pre- and post-transplant sample was needed to evaluate each patient at more than one time-point. Of the 18 patients positive pre-transplant, 12 converted to negative post-transplant within 30 days of transplant, and 2 took more than 90 days to convert to AVA negative. Four of the 18 patients remained positive post-transplant.

Time-point	Day -30 – Day +0	Day +0 – Day +30	Day +31 – Day +60	Day +61 – Day +90	>Day +91
AVA-	-	12	-	-	2
AVA+	18	1	1	-	2

Table 5. Time point post-transplant where patients AVA+ pre-transplant converted to AVA-. Fourteen patients converted to AVA negative within 12 months and 4 patients remained AVA+ post-transplant.

	AVA negative	AVA positive
CMR +(p=.97)	14	9
AMR+ (p=.24)	6	2

Table 6. Rejection and Pre-transplant AVA status. The distribution of AVA positivity and rejection status was not statistically significant in pre-transplant samples.

Lastly, six patients converted from AVA negative to AVA positive. Fifty-percent (3/6) of these patients had a rejection episode (2CMR, 1 AMR and CMR) within six months of AVA positivity, although AVA positivity could not predict an episode of cellular mediated rejection (CMR) nor antibody mediated rejection (AMR).

Conclusion and Discussion

This study supports the design of a successful Luminex multiplex assay, which has a higher detection of AVA than ELISA. A main concern when designing a Luminex-based serum assay is the signal-to-noise ratio. To maximize the test signal, sera were tested at a 1:25 dilution. In early experiments (data not shown) the NAB exhibited an unexpected elevated signal. In an attempt to reduce the test background two filter plate companies were evaluated, NAB sera were ultra-centrifuged and different wash buffer compositions were evaluated. We found tween-20 to be a necessary component in the wash buffer as it acts as a detergent preventing the beads from sticking to the filter plate.

Ultracentrifugation did not provide an additional decrease in background. Additionally, hypotonic dialysis is a procedure used to remove interfering factors from patient serum. This alternative to reducing background through hypotonic dialysis could not be performed because it would have resulted in removal of the target IgM antibody.[38] Due to the retrospective nature of the sera tested, the integrity of the IgM should be considered. [39] The stability of the IgM after repeat freeze thaw cycles coincides with a significant loss of reactivity on immunoassays. This indicates the need for a prospective study, which would provide better quality sera to determine if stronger signals could be achieved.

The test procedure was derived from the Single Antigen Bead Assay (One Lambda, Inc.) [40]. In the development of this assay the number of washes and incubation periods were not altered. Because the seroMAP technology on the Luminex platform has been established as achieving high sensitivity, we followed a standard protocol. In this regard we did not alter these variables.

The ELISA assay was considered to be the current standard and compared to the AVA Luminex assay. The two-fold increased sensitivity of the Luminex assay observed over the ELISA has previously been reported.[41] The competitive inhibition experiment revealed the sera positive by Luminex only, are indeed identifying vimentin specific antibody. This is suggestive that the sera positive by Luminex only are true positives not identified by the ELISA.

The Luminex assay has shown to provide reproducible, sensitive and specific results. A weakness in evaluating the presence of AVA in the target population was a lack of statistical power due to a small sample size. To power a statistically relevant conclusion between AVA Luminex positive sera and orthotopic heart transplant recipients, more sera will need to be tested, obtaining sera at all time points for all patients post-transplant. Additionally, an appropriate negative control group would need to be determined. This would eliminate relying solely on the NAB to access a negative sample.

The successful design of a Luminex multiplex assay for the detection of anti-vimentin antibody has clinical application.. A small study group (n=46) was evaluated and demonstrated an expected distribution of AVA, but the sample size did not provide the power needed to establish statistically relevant results. Although this assay was originally proposed for post-transplant monitoring of AVA, 18/46 patient's pre-transplant samples were positive for AVA. The presence of AVA could not correlate with CAV in this study; however, the presence of low-level antibody pre-transplant may be a predictor of long-term poor outcomes. Due to this, additional studies should focus on outcomes greater than 12 months. Of the 18/46 initially positive patients, 12/18 converted to negative post-transplant and 4/18 remained AVA positive. This turn to AVA negative may be a dilution effect from the number of transfusions these patients endure during the transplant process, equivalent to a whole body transfusion. Even though AVA was not found to be a predictor of CAV in this patient group, we found no difference in the distribution if patients were sensitized to HLA or experienced rejection; however, the AVA positivity in the presence of DSA was trending toward significance and should be considered in future studies. Additionally, it has also been shown with other auto-antibodies and minor histocompatibility antigens the deleterious effects are observed in the long term.

In conclusion, the Luminex multiplex assay was determined to be twice as sensitive as ELISA. Although high background can interfere with the AVA signal, reproducible, sensitive and specific results can be produced. To power a statistically relevant conclusion between the presence of AVA and CAV, a larger prospective study needs to be conducted. Even though near 40% of pre-transplant samples tested positive for AVA, long-term outcomes should be evaluated to determine if there is a need for post-transplant anti-vimentin monitoring.

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